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Isolation and genetic characterization of phenol-utilizing marine bacteria and their phenol degradation pathway

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Abstract: Phenolic compounds are widely distributed toxic pollutants in seawater, and their effective degradation is very important for bioremediation programs. In this study, nine phenol-degrading bacteria were isolated from seawater samples, which were collected from the coastal areas of Japan. Besides the enrichment substrate phenol, all isolates could utilize at least one isomer of cresol as the sole source of carbon. A 16S rRNA gene sequence analysis indicated that all strains were affiliated with the class *Gammaproteobacteria*, four strains were closely related to *Spongiibacter*, four were closely related to *Marinobacter*, and one was closely related to *Photobacterium*. During growth on phenol, all isolates produced a yellow product, and a whole-cell study indicated that it was an extradiol *meta*-ring cleavage product of catechol, 2-hydroxymuconate semialdehyde. Phylogenetic analysis revealed that the partial gene encoding the largest subunit of the multicomponent phenol hydroxylase of the isolates was similar to that of terrestrial bacteria, thereby suggesting that phenol is converted into catechol by marine bacteria. We also suggest that horizontal transfer of the gene may occur not only among marine bacteria but also between the genera *Marinobacter* and *Pseudomonas*.

Keywords: Phenol Degradation, Marine Bacteria, Multicomponent Phenol Hydroxylase, Catechol 2, 3-Dioxygenase, Isolation, *Marinobacter*, *Spongiibacter*, *Photobacterium*

1. Introduction

Phenolic compounds are widely distributed toxic pollutants in the environment, which are produced from a variety of industries and natural sources [1, 2]. Phenolic compounds in the soil flow into rivers because of their high water solubility and rivers flow into the sea; this is the reason for the increase in the concentration of phenolic compounds in the sea. Furthermore, crude oil spills resulting from tanker accidents, submarine and offshore oil field accidents, oil pipeline accidents, etc., also increase the concentration of phenolic compounds in the sea. Therefore, clarifying the mechanism of phenol biodegradation in seawater and assessing the ability to degrade phenol in the marine environment are very important for developing effective bioremediation programs.

A number of phenolic compound-degrading microorganisms have been isolated, and their degradation pathways and genes for degrading phenolic compounds have been studied in detail [2, 3]. However, these microorganisms have mainly been isolated from terrestrial or freshwater sites. *p*-*n*-nonylphenol-degrading marine bacteria have been isolated from seawater obtained from coastal areas of Japan; however, these bacteria cannot utilize phenol and cresol as their sole sources of carbon [4, 5]. A phenol-degrading marine bacteria *Marinobacter* sp. was recently isolated from a harbor in South Africa [6]. However, information regarding phenol-degrading bacteria from marine environments is relatively scarce and there is no information about phenol-degrading genes from marine bacteria. Therefore, more information regarding marine phenol-degrading bacteria and their degradation pathways is required to facilitate the development of effective bioremediation programs. In this paper, we report the isolation of new marine bacteria that utilize phenol as the sole source of carbon, and an analysis of a partial gene from the isolates that encodes the largest subunit of the multicomponent phenol hydroxylase (LmPH).

2. Materials and Methods

2.1. Isolation of Phenol-Degrading Bacteria from Seawater

We collected 10 L of surface seawater from each of five coastal areas of Japan: Chiba, Nagasaki, Okinawa, Fukui, and Ogasawara islands in Tokyo. Marine bacteria were collected from each seawater sample by filtration and resuspended in 30 mL of commercial artificial seawater medium Daigo's IMK-SP (Nihon Pharmaceutical, Osaka) [7]. Enrichment and purification of the strains were performed according to a previously described procedure with some modifications [7]; Daigo's IMK-SP medium was supplemented with 5 mM phenol.

2.2. Utilization of Cresols for Growth

The ability of isolated strains to grow on cresol as the sole source of carbon was determined using Daigo's IMK-SP containing 3 mM *o*-, *m*-, or *p*-cresol respectively at 25°C for 10 days. Daigo's IMK-SP containing 3 mM phenol was used as a positive control of growth and Daigo's IMK-SP was used as a negative control.

2.3. Polymerase Chain Reaction and Sequencing Analysis

16S rRNA gene sequences were determined as previously described [8]. Phylogenetic trees were generated by the neighbor-joining method in MEGA version 6.0 [9]. To amplify the approximately 930-bp long partial gene encoding catechol 2,3-dioxygenase, we used two previously described degenerate primers [10]: C23O-ORF-F, AGGTGWCGTSATGAAMAAAGG and C23O-ORF-R, TYAGGTSAKMACGTTCAKGAA. To amplify the approximately 620-bp long partial gene encoding LmPH, we used two previously described degenerate primers [11]: pheUf, CCAGGSBGARAARGAGARGAARCT and pheUr, CGGWARCCGCGCCAGAACCA. PCR (Polymerase chain reaction) amplifications were performed using BLEND *Taq* -Plus- (Toyobo) in a PCR Thermal Cycler Dice (Takara Bio) under the following conditions: 2 min at 94°C, followed by 30 cycles for 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C. DNA sequences of the amplified fragments were determined by directly sequencing with PCR primers. The nucleotide and protein sequence similarity searches were performed using the BLAST program on the NCBI website [12]. Sequence identity values were calculated using GENETYX-MAC version 16 (Genetyx Corporation). The nucleotide sequences determined in this study were deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB985579–AB985596 and LC012309.

2.4. Whole Cell Studies

The medium used for preparing whole cells was the same as that described above for strain isolation. Cells grown on phenol were harvested in mid-exponential phase by centrifugation at 10,000 ×*g* for 10 min at 4°C and washed twice with 21 mM sodium–potassium phosphate buffer (pH 7.1) supplemented with 2.0% NaCl. The cells were resuspended in the buffer to adjust the optical density at 600 nm to 5.0, incubated with 2.0 mM phenol in a rotary shaker at 25°C, and the spent buffer was scanned using a VIS/UV spectrophotometer U-3010 (Hitachi High-Technologies, Tokyo).

3. Results and Discussion

Bacterial strains that grew on phenol as the sole source of carbon were isolated from all five sampled areas (Table 1). Besides the enrichment substrate phenol, all strains could utilize *o*-, *m*-, or *p*-cresol as the sole source of carbon (Table 1). These results indicate that the ability to degrade phenol and cresol is widely spread in the marine environment.

Table 1. *Phenol-degrading isolates from seawater*

Source	Strain	16S rRNA gene sequence analysis		Ability of the isolated strains to grow on:		
		Closest species in database (Acc. No.)	Identity (%)	*o*-cresol	*m*- cresol	*p*- cresol
Chiba	KU17C	*Marinobacter litoralis* (AF479689)	100	+	+	+
Nagasaki	KU17D	*Spongiibacter marinus* (AM117932)	99.7	+	−	−
Okinawa	KU17E1	*Marinobacter algicola* (AY258110)	98.2	+	+	+
Okinawa	KU17E2	*Photobacterium gaetbulicola* (GQ260188)	96.6	−	−	+
Fukui	KU17F2	*Spongiibacter marinus* (AM117932)	99.9	+	−	+
Fukui	KU17F4	*Marinobacter hydrocarbonoclasticus* (AB021372)	97.6	+	+	+
Tokyo (Ogasawara islands)	KU17G1	*Spongiibacter marinus* (AM117932)	99.9	+	−	+
Tokyo (Ogasawara islands)	KU17G2	*Spongiibacter marinus* (AM117932)	99.8	+	−	+
Tokyo (Ogasawara islands)	KU17G3	*Marinobacter hydrocarbonoclasticus* (AB021372)	98.5	+	+	+

The isolated bacteria were characterized by 16S rRNA gene sequence analysis (Table 1, Figure 1). We found that all isolates were phylogenetically affiliated with the class *Gammaproteobacteria*, and the isolates were most closely related to the genera *Marinobacter, Spongiibacter*, and *Photobacterium*. For the first time, members of the genera *Spongiibacter* and *Photobacterium* were shown to be capable of utilizing phenol. The majority of the isolates were affiliated with *Marinobacter* and *Spongiibacter*: four *Marinobacter* and four *Spongiibacter*. This observation suggests that members of the genera *Marinobacter* and *Spongiibacter* are widely distributed in the sea as phenol degraders. In particular, our finding of *Marinobacter* is supported by the previous isolation of a phenol-degrading *Marinobacter* in marine water from

South Africa [6]. Members of the genus *Marinobacter* have been reported to be present in a large variety of ecosystems, ranging from extremely cold to hot environments, and they tolerate a broad range of salinity and pH, thereby demonstrating their high adaptive capacity [13]. Furthermore both culture-dependent and -independent methods have indicated that members of the genus *Marinobacter* are dominant in oil-contaminated marine environments, and these studies have indicated that members of the genus *Marinobacter* play an important role in the degradation of several organic compounds in marine environments [14-18]. Together with these reports, our results suggest that members of the genus *Marinobacter* also play an important role in phenol degradation in several marine environments; thus, they have tremendous potential for applications in the bioremediation of phenol-contaminated marine and saline environments.

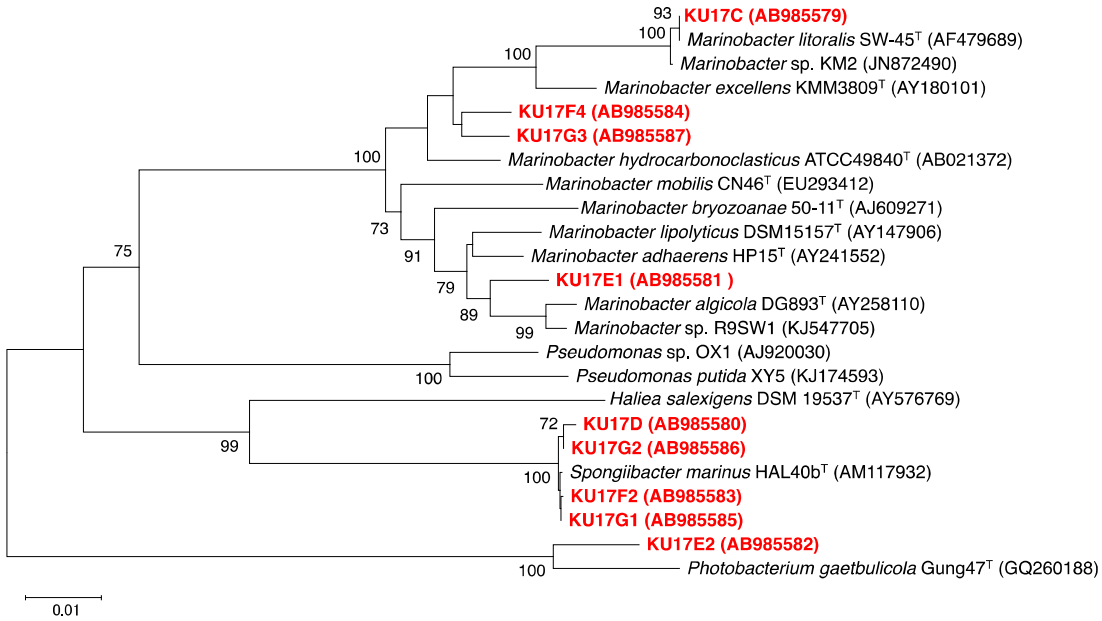

Figure 1. *A neighbor-joining tree showing the phylogenetic position of phenol-degrading isolates and their related species based on 16S rRNA gene sequences. The GenBank accession number for each microorganism used in the analysis is shown in parentheses after the species name. Bootstrap values (expressed as a percentage of 1,000 repetitions) >70% are shown at the branch. Bar, 0.01 substitutions per nucleotide position.*

During growth on phenol, all isolated strains produced a yellow product, which was probably the *meta*-ring cleavage product 2-hydroxymuconate semialdehyde. To clarify the formation of 2-hydroxymuconate semialdehyde, whole cell studies were performed using the selected strain KU17C. Whole cells of strain KU17C were incubated with phenol and the supernatant was examined using a VIS/UV spectrophotometer (Figure 2). The decrease in absorption at 270 nm indicated the degradation of phenol and the increase in absorption at 375 nm indicated the formation of 2-hydroxymuconate semialdehyde, as a result of the extradiol *meta*-ring cleavage of catechol by catechol 2,3-dioxygenase [19]. A yellow product with maximum absorption at 375 nm was also produced from catechol by the phenol-grown whole cells of strain KU17C (data not shown). Furthermore, PCR amplification using C23O-ORF primers produced the expected ca 930-bp DNA fragment. The deduced amino acid sequence of the amplified fragment was 78.8% identical to that of the well-characterized catechol 2,3-dioxygenase of *Pseudomonas* sp. strain OX1. This high identity strongly suggests that the amplified DNA encodes catechol 2,3-dioxygenase, which supported our finding that catechol is cleaved by *meta*-cleavage in strain KU17C. The highest score in the BLASTP search was a putative catechol 2,3-dioxygenase from *Haliea salexigens* strain DSM 19537 with an identity of 98.9%. These results showed that marine phenol-degrading bacteria metabolize phenol via the *meta*-cleavage pathway, which is catalyzed by two key enzymes: phenol hydroxylase and catechol 2,3-dioxygenase.

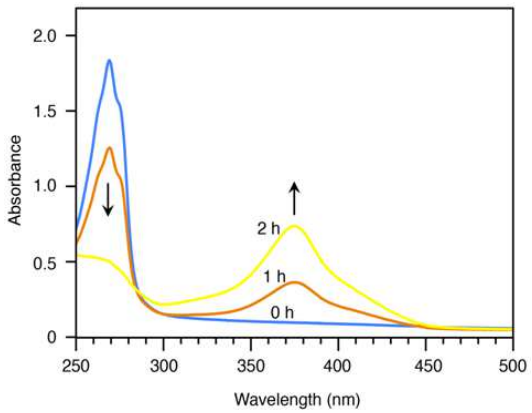

Figure 2. *Spectral changes during conversion of phenol by whole cells of strain KU17C grown on phenol. The reaction mixture was scanned at 0 h, 1 h, and 2 h. Arrows indicate direction of spectral change.*

It has been suggested that multicomponent phenol hydroxylases (mPHs) are dominant in the environment and

that mPHs among terrestrial bacteria are highly similar [20]. From our conclusion that marine phenol-degrading bacteria metabolize phenol via the same pathway as terrestrial bacteria, and from other reports [20], we hypothesize that marine bacteria also possess genes that encode mPHs. To test this hypothesis, we used PCR to screen for possible genes encoding LmPH in the genomic DNA of isolates, and then performed a phylogenetic analysis of these genes (Figure 3). As expected, an approximately 600-bp DNA fragment was amplified from the genomic DNA of all the isolates (data not shown). The nucleotide sequence and the deduced amino acid sequences of the amplified fragments were 75.1%–88.0% and 79.8%–96.7% identical to the LmPH of the well-characterized mPH of *Pseudomonas* sp. strain OX1 (Table 2) [21-25], respectively. These high identities strongly suggest that the amplified DNA sequences encode LmPH. Among the isolates, the deduced amino acid sequence identities of the amplified fragments ranged from 79.8%–100%. These high similarities suggest that the genes encoding LmPH in these isolates and in *Pseudomonas* sp. strain OX1 were derived from the same origin, but varied over a long time, and thus phenol utilization by the marine isolates and terrestrial bacteria involves a common mechanism.

Table 2. *Identities of the nucleotide sequence and the deduced amino acid sequence of putative LmPH genes from isolates compared with those of LmPH genes from Pseudomonas sp. strain OX1*

Strain	Nucleotide sequence identity (%)	Amino acid sequence identity (%)
KU17C	85.8	93.4
KU17D	80.8	95.6
KU17E1	84.9	94.5
KU17E2	75.1	79.8
KU17F2	80.6	95.6
KU17F4	87.7	95.1
KU17G1	80.9	95.6
KU17G2	81.9	95.6
KU17G3	88.0	96.7

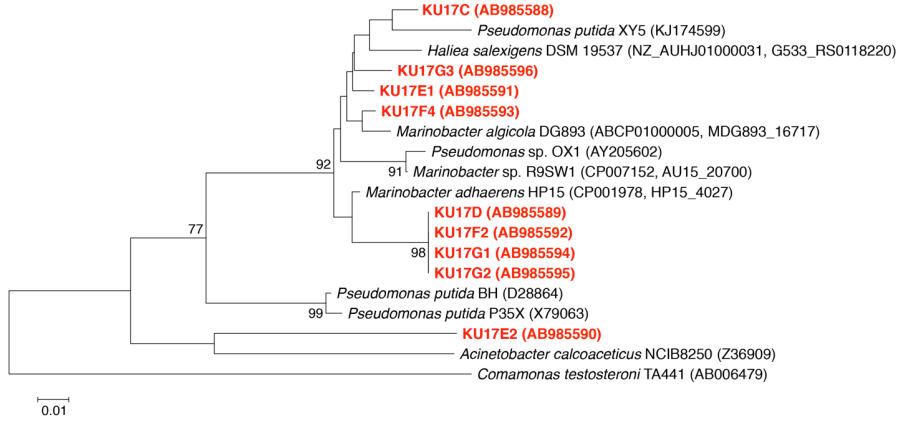

Figure 3. *Neighbor-joining tree showing the phylogenetic position of the nucleotide sequence of genes encoding the putative partial LmPH. The GenBank accession number for each gene is shown in parentheses after the species name. Bootstrap values (expressed as a percentage of 1,000 repetitions) >70% are shown at the branch. Bar, 0.01 substitutions per nucleotide position.*

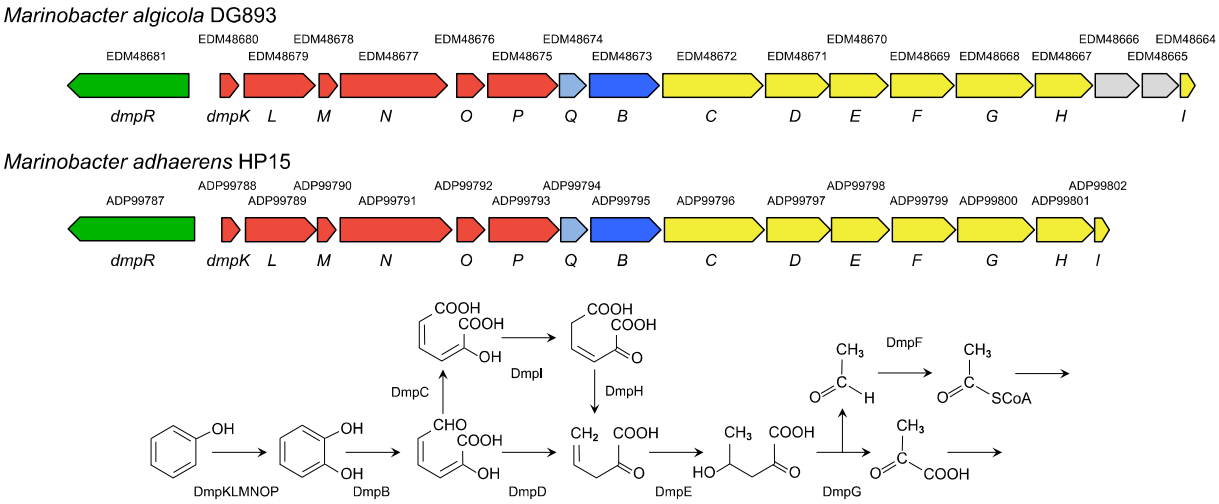

Figure 4. *Organization of putative phenol degradation gene clusters in the genome sequences of Marinobacter algicola strain DG893 and Marinobacter adhaerens strain HP15, and proposed phenol degradation pathway. DmpR, NtrC family transcriptional activator; DmpKLMNOP, components of phenol hydroxylase; DmpB, catechol 2,3-dioxygenase; DmpC, 2-hydroxymuconic semialdehyde dehydrogenase; DmpD, 2-hydroxymuconic semialdehyde hydrolase, DmpE, 2-oxopent-4-dienoate hydratase; DmpF, acetaldehyde dehydrogenase; DmpG, 4-hydroxy-2-oxovalerate aldolase; DmpH, 4-oxalocrotonate decarboxylase; DmpI, 4-oxalocrotonate isomerase; and DmpQ, ferredoxin-like protein involved in reactivation of catechol 2,3-dioxygenase.*

BLASTP searches revealed that two *Marinobacter* genomes, i.e., those of *Marinobacter algicola* strain DG893 (accession number: ABCP01000005) and *Marinobacter adhaerens* strain HP15 (accession number: CP001978) contain a gene encoding a putative LmPH, and the remaining phenol *meta*-cleavage pathway genes are located in the DNA region adjacent of the gene (Figure 4) [26, 27]. This supports our finding that *Marinobacter* spp. degrade phenol via the *meta*-cleavage pathway. BLASTP searches also showed that the deduced amino acid sequence of the LmPH amplified from strain KU17C exhibited the highest score when compared with the deduced amino acid sequence of the putative LmPH gene of *Haliea salexigens* strain DSM 19537, with amino acid sequence identity of 97.8% and nucleotide sequence identity of 91.3%. Although protein-encoding genes have been reported to vary much faster than rRNA-encoding genes [28], the divergence in the 16S rRNA gene sequences of strain KU17C and *Haliea salexigens* strain DSM 19537 (12.8%) was higher than the divergence in their LmPH gene sequences (8.7%). Similarly, the putative LmPH gene of the terrestrial *Pseudomonas putida* strain XY5 was only 2.4% divergent with strain KU17C, and the putative LmPH gene of *Marinobacter* sp. strain R9SW1 was only 4.5% divergent with the LmPH gene of *Pseudomonas* sp. strain OX1. The divergence rates in the 16S rRNA gene sequences were 10.6% and 10.2%, respectively. These results suggest that LmPH genes were horizontally transferred among these bacterial lineages. The horizontal transfer of an aromatic compound-degrading gene between the genera *Marinobacter* and *Pseudomonas* has also been suggested for the naphthalene-degrading *Marinobacter* sp. strain NCE312 [29].

4. Conclusion

In this study, we successfully isolated nine phenol-utilizing marine bacteria, which were most closely related to the genera *Marinobacter, Spongiibacter*, and *Photobacterium*. PCR screening revealed that all isolates possessed a gene that encoded putative LmPH, which was similar to that found in terrestrial bacteria. The sequence data for LmPH-encoding genes analyzed in this study should facilitate the development of informative genetic markers to evaluate the efficacy of bioremediation in phenol-contaminated marine and saline environments. Further genetic analysis will provide more useful information to support the development of the genetic markers and the improvement of the phenol biodegradation potential of the isolated strains, which may contribute to phenol bioremediation processes in various saline ecosystems, although these studies are outside our present scope.

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